AN ATTENUATED CULTURE OF TRYPANOSOMA BRUCEI *

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SYNOPSIS

INTRODUCTION

The production of active immunity may be brought about by the introduction into the animal body of living organisms either fully virulent or attenuated, or dead organisms or their products. The injection of virulent organisms either in sublethal dose, or into parts of the body not used by the organism as a channel of entrance in the natural infection, is uncertain and often dangerous. On the other hand, the injection of dead organisms, though not usually attended with danger, is often ineffectual because with the death of a germ, depending on the method of treatment, the antigen or immunity-producing substance may be partially destroyed. The introduction of a non-virulent or slightly virulent organism would then, *a priori,* suggest itself as the most suitable for the production of immunity.

It may be well to mention that attenuation may be produced in various ways, that is, by treatment of organisms with chemical substances; by exposure to moderately high temperature, or to the action of light; by cultivation in immune serum; by passage through relatively insusceptible animals; and finally, by long-continued cultivation outside of the animal.

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Protection against experimental infection by the various organisms may be obtained by application of one or more methods of immunization referred to. This is particularly true of the bacterial diseases, which for obvious reasons, have claimed the most attention in the past. On the other hand, with very few exceptions, very little has as yet been done in the immunity against protozoal diseases. This has been due chiefly to the fact that pure cultures of the pathogenic protozoa have not been available. With the successful cultivation of certain trypanosomes it has been possible to approach this problem along experimental lines. Among the strictly pathogenic flagellates, the trypanosoma brucei has thus far been cultivated in vitro for many generations.

The problem which presented itself was to ascertain whether or not the cultures of this protozoan could be rendered avirulent or attenuated, and further, whether such modified cultures would be capable of conferring immunity. This paper deals only with the question of attenuation produced by long continued cultivation of this organism. As will be shown in the following pages, this result has been in part accomplished, for while our modified strain is still somewhat pathogenic for susceptible animals, it has practically lost its pathogenic power for others.

Attenuation of this organism, then, would suggest the possibility of obtaining similar results with the other pathogenic trypanosomes when they are successfully cultivated.

ATTENUATION OF THE TRYPANOSOMA LEWISI

The first demonstration of the attenuation of a cultural trypanosome was made by Novy, Perkins and Chambers.[1] The organism studied was trypanosoma lewisi of a rat. Their culture isolated in September, 1905, at first produced typical infection in rats. After a long interval the culture was again tested as to its virulence in March, 1909, and after numerous trials it was found that it did not produce infection, and in fact, had become completely attenuated. The organism was at this time in its one hundred and fifty-eighth generation. Since a considerable interval had elapsed, in which the virulence was not tested, it could not be determined whether the loss of virulence was gradual or quite sudden. Neither could it be determined just how early after isolation such loss had occurred.

1. *Jour. Infect. Dis.*, 1912, 11, p. 411.

It was desirable, accordingly, to confirm this work on another strain of trypanosoma lewisi, and at the same time to ascertain just how early evidence of attenuation could be obtained. Accordingly a culture of trypanosoma lewisi was isolated in October, 1911. This was maintained by weekly transplants on a blood agar medium consisting of equal parts of defibrinated rabbit's blood and nutrient agar. These cultures were grown at a temperature of approximately 25 C. They invariably gave very rich growth, which at times could be seen as whitish colonies not unlike those of bacteria. These cultures produced typical infection in rats for a considerable period after isolation. Evidence of attenuation first appeared in the fifty-first generation, which culture on injection into two rats produced typical infection in one, and only a slight infection of rather short duration in the other (see Table 1).

TABLE 1.

RESULTS OF INOCULATION OF CULTURES OF THE TRYPANOSOMA LEWISI.

No. of Rat	Generation of Culture	Period of Incubation Days	Duration of Infection Days	Remarks
2.25	51	2	12	Examination discontinued 12 days after inoculation
3.25	51	2	9	The number of organisms seen was never more than 3 per field
3.118	59	4.5	—	On the ninth day after inoculation when the number of organisms was 25 per field the rat was bled
4.118	59	—	—	No infection
1.67	65	5-6	1	2 organisms seen
2.67	65	6-7	3	⅕ per field, 3, 1 organism seen
9.149	69	3-5	1	2 organisms seen
10.149	69	—	—	No infection
3.190	71	—	—	No infection
4.190	71	—	—	No infection
4.104	75	5-6	4	One organism seen
5.104	75	—	—	No infection

A second test, made with the fifty-ninth generation, showed even more marked attenuation. Of two rats inoculated one showed no organisms in its blood, while the number in the other never reached more than twenty-five per field (No. 7 objective).

The third test, with the sixty-fifth generation, gave still better evidence. In one of the two rats inoculated trypanosomes were found only on the sixth day, at which time but two could be detected. In the other rat the parasites appeared on the seventh day, when they were about 0.2 per field. On the eighth day only three and on the ninth only one organism could be found.

A test of the sixty-ninth generation gave in one rat a transient infection, which lasted but one day. Only two organisms were found, while its companion failed to show any parasites.

The inoculation of the seventy-first generation into two rats yielded no infection.

The last test, made with the seventy-fifth generation, gave a negative result in one rat, and a mild infection of very short duration in the other.

In all the tests above mentioned the rats used for the experiment were young, and a natural infection was excluded by daily examination for at least two weeks before they were used in an experiment. In each test a rat was given an intraperitoneal injection of the contents of one cultural tube, which was grown for seven days at 25 C. After the injection the rats were examined as a rule every day for at least two weeks.

While confirming the production of attenuation, the fact is established that the loss of virulence becomes evident in fifteen months, a period represented by sixty or more generations.

CULTURE OF TRYPANOSOMA BRUCEI

It is well known that trypanosoma brucei was first cultivated by Novy and MacNeal in 1903.[2] The medium used was defibrinated blood agar similar to that employed in the previous successful cultivation of the trypanosoma lewisi.[3] The agar was prepared by adding to the meat extract (1-8) 2 per cent. peptone, 0.5 per cent. sodium chlorid, 1 per cent. normal sodium carbonate, and 2 per cent. agar. One part of this agar was mixed with two parts of defibrinated rabbit blood, and the mixture solidified in an inclined position. The medium was inoculated with two drops of defibrinated rat blood, very rich in trypanosomes. At 25 C. growth took place in the water condensation, and no visible colonies could be made out. Of fifty animals thus tested Novy and MacNeal found that only four gave positive results. Smedley[4] found that three out of ten attempts were positive.

Because of the inconsistent results it seemed advisable to attempt an improvement of the medium. Various attempts were made in this direction. The best results were obtained by the employment of a dialyzed meat extract, which was made as follows: 125 gm. chopped beef, and 250 c.c. of water were allowed to digest over night in the cold, or for one hour at 55 C. The mixture was then strained and the extract boiled and filtered. The filtrate was then dialyzed in a large collodium sac against running distilled water for twenty-four to

2. *Jour. Amer. Med. Assn.*, 1903, 41, p. 1266; *Jour. Infect. Dis.*, 1904, 1, p. 1.
3. Contributions to Med. Research Dedicated to V. C. Vaughan, 1903, p. 549.
4. *Jour. Hyg.*, 1905, 5, p. 38.

forty-eight hours. The dialyzed sac contents were diluted to 1 liter with distilled water, and then 2 per cent. peptone, 0.5 per cent. sodium chlorid, .01 per cent. calcium chlorid, 1 per cent. normal sodium carbonate, and 2 per cent. agar added. About 1 c.c. of this agar was placed in each tube and sterilized in an autoclave by heating to 105 to 108 C. for fifteen minutes. Shortly before use the desired number of agar tubes were melted in the water bath, cooled to 60 C., and two volumes of defibrinated rabbit blood were added. The mixture was well agitated, and then allowed to solidify in a slanting position.

A number of experiments were made with a medium in which the meat extract was replaced by an extract made of peas and beans, and obtained by boiling 1 per cent. of each with distilled water. To this extract the usual amounts of sodium chlorid, alkali, and agar were added. On the addition of two parts of defibrinated rabbit blood a medium was obtained which gave more constant results than those obtained with the original meat extract (1 to 8).

A third medium was employed similar to that employed by Nicolle.[5] In this no meat extract was employed. It was prepared by dissolving 2 per cent. agar, 2 per cent. peptone, and 0.5 per cent. sodium chlorid in distilled water, no alkali being added. It was diluted with two volumes of defibrinated rabbit blood, the same as in the case of the other media.

In order to ascertain the relative value of these media as compared with the original medium of Novy and MacNeal a series of cultures were carried out. For this purpose the blood of an infected rat was transferred to not less than six, usually twelve tubes of each media, and incubated in the usual way at 25 C. Six comparative trials were thus made. The results of these tests were decidedly favorable to the dialyzed meat extract medium, since 80 per cent. of the tubes with this medium gave a positive growth. In the case of the pea and bean medium 53 per cent. of the tubes were positive. The modified medium of Nicolle gave about the same result, namely, 48 per cent. of successful cultures. The least favorable results were obtained with the original medium, since only 25 per cent. of these were successful.

No advantage was found by altering the amount and kind of alkali in these media. It was hoped to secure better results by keeping the inoculated tubes in atmospheres of different gases, such as hydrogen,

5. *Arch. de l'Inst. Pasteur de Tunis,* 1908, p. 55; *Ann. de l'Inst. Pasteur,* 1909, 23, p. 361.

nitrogen and carbon dioxid, but all such attempts proved very unsatisfactory, and we found it best to adhere to the ordinary aerobic conditions.

Having a satisfactory nutrient agar, attempts were made to improve the blood constituent of the medium. For this purpose defibrinated rabbit blood was centrifugated, and the serum drawn off and diluted to the original blood volume with 0.5 per cent. sodium chlorid solution. The clear serum was then mixed with the dialyzed nutrient agar, in a ratio of two to one. The red blood cells, freed from serum, were likewise diluted with salt solution to the original blood volume, and this suspension added to the nutrient agar (2 to 1). On inoculation of these two media with trypanosomal blood it was found that the serum agar gave practically 100 per cent. successful cultures, whereas the medium containing only red blood-cells gave but 38 per cent. Inactivation of the serum for one-half to one hour gave essentially the same results as the diluted serum agar. It would seem advisable, therefore, in attempting isolation of trypanosoma brucei to employ dilute serum rather than whole defibrinated blood. We have found that this organism maintains itself without any difficulty on this serum medium, although no hemoglobin is present. In fact the growth on this medium becomes extremely rich, and is easily visible to the eye. For ordinary purposes the blood agar medium has been used since it is more easily prepared.

To obtain an initial culture it is advisable to employ the blood of a rat which is in the early stage of the infection, having from ten to thirty-five trypanosomes per field. The results do not seem to be as good when the inoculation is made with blood obtained from the later stages of the disease, when the parasites are more numerous. In the inoculated tubes the cultural forms of trypanosomes may occasionally be seen as early as the sixth day. They are more likely to appear on the twelfth or fourteenth day, and exceptionally they may be delayed as late as twenty-one days.

After obtaining such excellent results with nagana it would seem that this medium could be used for the cultivation of the other pathogenic trypanosomes. This expectation, however, was not realized, and although many attempts were made to cultivate the trypanosomes of sleeping sickness, caderas, surra, and dourine, they were invariably unsuccessful.

The culture used in the present work was isolated on March 15, 1910, on the pea and bean blood medium. After four generations (weekly transplants) on this medium, it was transplanted to dialyzed nutrient blood agar, and since this gave a richer growth the culture has been maintained from that time on the latter. The blood used in these cultures was drawn from the carotid artery of a rabbit under aseptic conditions, and immediately defibrinated. It was then drawn up into a bulb, and placed in the ice chest for three to four days before use. It was found that such blood was more suitable than blood freshly drawn and immediately used. Furthermore, tubes inoculated immediately after slanting did not give as good a growth as those kept in the ice chest for a day. The transplants were made by means of a pipet instead of the loop used in the transplantation of the trypanosoma lewisi.

At present (Jan. 1, 1914), the culture is in its one hundred and ninety-third generation, and shows no indication of degeneration or exhaustion; in fact, it is in a most excellent condition, and invariably yields a very rich growth, which at times is evidenced by a faint whitish film on the surface of the medium. (On July 3 the culture is in the two hundred and sixteenth generation.)

MORPHOLOGY OF THE CULTURAL TRYPANOSOMES

In the cultures the trypanosomes occur either as single, free-swimming cells, or in groups consisting of a varying number of organisms, from five to six to as many hundreds. These groups at times attain great size, filling up the field (No. 7 objective). The groups do not have the regular symmetrical arrangement of the cultural rosettes of the trypanosoma lewisi, but present the picture of a disorderly, writhing mass similar to a Medusa head. At times, however, fairly symmetrical rosettes occur that bear a striking resemblance to a "sunburst." In all these groups the flagella are directed outward. There is a marked variation in the cultural forms, short, stubby spindle forms being found side by side, with long thin ones, the latter preponderating. The organisms often occur in pairs attached at their posterior ends. An undulating membrane, though present, is not in any way conspicuous, as in the blood forms; this is due to the fact that the micronucleus, as a rule, is situated anterior to the nucleus.

In cultures grown on the ordinary blood-agar the cells show, instead of a homogeneous cytoplasm, one or two highly refractive globules which may be of considerable size. In the later generations, espe-

cially when grown on the dialyzed blood-agar medium, the globules become smaller and more numerous, and not infrequently cells entirely devoid of these granules have been found. The presence of large intracellular globules may probably be considered as pointing to a somewhat unfavorable medium, and their decrease or absence would indicate an adaptation of the trypanosomes to their environment, or at least a decided improvement in the quality of the medium.

The transplantation of the culture after many generations on blood medium to serum agar gives a good growth which is often heavier than that in blood agar, and is readily seen as a white scum on the surface of the medium. The fact that the organism can be maintained indefinitely on the serum-agar medium would indicate that the presence of hemoglobin is not necessary to the culture of the trypanosoma brucei. In this connection it may be of interest to state that transplantation from the blood-agar culture to ascitic-fluid agar was entirely unsuccessful in three separate attempts.

EFFECTS ON ANIMALS

Novy and MacNeal[6] were the first to try the effect of inoculations of cultures into animals. They found that the survivals in tubes, in which no multiplication had taken place, infected rats and mice on the fifth and even on the ninth or tenth day, although failures to infect were noted in two tests with eight-day material. When the cultures of the organism were obtained these were found to be almost as virulent as the rat strain of the virus. The intraperitoneal injection of virulent cultures (Generations 2-5) was found to produce death in mice and rats at times in three and one-half days, usually in seven or eight days, and exceptionally as late as eleven days. The period of incubation in the case of the animals infected with such cultures varied from three, seven or nine days, but in most cases organisms were first seen on the fifth day after inoculation. Since the intraperitoneal injection of virulent blood usually killed rats and mice in from three to five days, it would seem as if this culture, even in early generations, had already undergone partial attenuation.

The results of Novy and MacNeal's experiment further showed that the age of the culture, temperature at which the tubes were kept and composition of the medium greatly influenced the virulence of the organisms. Tubes incubated at 25 C. and then transferred to 34 C. for

6. *Jour. Amer. Med. Assn.*, 1903, 41, p. 1266; *Jour. Infect. Dis.*, 1904, 1, p. 1.

one or two days quickly lost their virulence for rats and mice. As regards the effect of age, they found that cultures which were 18 to 20 or more days old were usually non-infective. Their views, that cultures when kept for from three or four weeks at 25 C. will lose their virulence, has been confirmed by the results which will be given later.

Smedley[7] carried the trypanosoma brucei through three generations on the Novy-MacNeal medium. He failed to produce an infection in one rat and three mice, the cultures used being either first, second or third generation. Smedley explains this by stating that the cultures were probably too old, and that possibly the amount of material injected was too small. He injected into the mice one or three loopsful, and into the rat 1 c.c. of the material. The age of the cultures injected was 15, 25, 28 and 32 days. It will be shown later that Smedley's negative results were undoubtedly due to the fact that the cultures were too old.

Our own experiments with cultures have fully confirmed the fact that they are infective for the ordinary laboratory animals. This is particularly true for the early generations. In order to demonstrate the loss of virulence, if any, by prolonged cultivation, it was necessary to make tests at long intervals of time. Inasmuch as there was reason to believe that no material loss of virulence occurred during the first year of cultivation the animal inoculations were not made with any regularity until after that time. Before going into a detailed consideration of these tests, it is desirable to indicate the method employed.

The cultures, unless otherwise indicated, were always grown at 25 to 27 C. for seven days. The tubes employed were 12 to 15 mm. by 150 mm. The medium which has been used almost from the first was prepared with the dialyzed meat extract, already mentioned. Before using a culture for injection, it was examined to ascertain its condition. At the end of seven days the tubes invariably showed an abundance, in fact, an extremely rich growth, consisting of perfectly formed, very actively motile flagellates.

By means of a sterile, drawn-out tube pipet, about 1 c.c. of sterile salt solution (0.85 per cent.) was introduced into the tube and the growth then taken up and transferred to a sterile tube. This suspension was invariably taken up in a sterile syringe and injected intraperitoneally into the animal. In order to avoid any deleterious action of the ordinary distilled water, the salt solution was always made up with glass-redistilled water. Since even this solution might have some

7. *Jour. Hyg.*, 1905, 5, p. 40.

injurious action on the organisms, special care was taken to effect the transfer from the culture tube to the animal in the shortest possible time. As a rule, when only a single culture was injected, this did not require more than two minutes.

INOCULATION OF RATS

The white rat is extremely susceptible to trypanosoma brucei, for even a single trypanosome is capable of producing a rapidly fatal infection. The period of incubation, following the intraperitoneal injection of a small dose (0.1 to 0.01 c.c.) of the blood is very short, the parasite appearing in the blood of the inoculated rat within twenty-four, or at most forty-eight hours, and death occurs on the fourth or fifth day. The rat is, therefore, an excellent reagent for testing the virulence of the cultured trypanosome, and for that reason has been utilized more than any other animal in our work.

The inoculation of the first generation of the trypanosoma brucei gives variable results, depending largely on the age of the material. It may be assumed that the organism in this first generation goes through a sort of a transition stage, while accommodating itself to its new environment. The new cultural form which develops from the blood type is necessarily not as abundant as in the subsequent generations. If such a culture is injected in the early stage of its production it may possibly contain survivals of the original blood forms, and the infection may be due to such. On the other hand, if it is injected after three weeks, the cultural forms present may become so enfeebled by the prolonged action of temperature and medium as to be incapable of producing an infection. On examination of Table 2 it will be seen that all the rats (eleven) inoculated with the first generation, between the fifteenth and the fortieth day of cultivation, survived, and at no time showed flagellates in their blood. Of the thirteen rats inoculated with the first generation on the sixth to the eleventh day, nine became infected after a period of incubation of from four to six and eight days, and died on the eighth to the sixteenth day, whereas four were negative.

The second and subsequent generations do not show the variations noted in connection with the first generation, as the trypanosome has adapted itself to the new living conditions. The cultures develop promptly and are invariably rich at the end of seven days. The injection of a single tube of such a culture in the second generation, as

TABLE 2.

INOCULATION OF RATS WITH CULTURES OF TRYPANOSOMA BRUCEI GROWN AT 25 C.*

No. of Rat	Culture		Period of Incubation, Days	Death, Days	No. of Rat	Culture		Period of Incubation, Days	Death, Days
	Generation	Age, Day				Generation	Age, Days		
1	1	6	6-8	12‡	57	92	7	4-7	42
2	1	10	4-6	§	58	92	7	4-7	24
3	1	10	‖	—	59	93	7	4-7	13
4	1	10	‖	—	60	93	7	4-7	12
5	1	10	6-8	16	61	130	7	6-7	22
6	1	10	6-8	16	62	130	7	6-7	20
7	1	11	4-6	8	63	131	7	3-6	16
8	1	11	4-6	14	64	131	7	3-6	16
9	1	11	4-6	8	65	136	7	2-6	31
10	1	11	4-6	14	66	136	7	2-6	53
11	1	11	4-6	14	67	137	7	2-6	40
12	1	11	‖	—	68	137	7	2-6	28
13	1	11	‖	—	69	138	7	2-6	31
14	1	15	‖	—	70	138	7	2-6	64
15	1	20	‖	—	71	139	7	6	30
16	1	20	‖	—	72	139	7	6	27
17	1	20	‖	—	73	140	7	6	30
18	1	21	‖	—	74	140	7	6	30
19	1	21	‖	—	75	144	7	6-7	29
20	1	28	‖	—	76	144	7	8-9	28
21	1	29	‖	—	77	144	7	6-7	26
22	1	32	‖	—	78	150	7	5-7	69
23	1	40	‖	—	79	150	7	9-11	60
24	1	40	‖	—	80	152	7	6-8	§
25	2	7	3-4	7	81	152	7	6-8	38
26	2	7	5-6	9	82	153	7	5-7	§
27	2	7	6-7	10	83	153	7	5-7	60
28	2	7	5-6	9	84	154	7	8-10	126
29	2	7	4	9	85	154	7	8	¶
30	32	7	4-5	§	86	154	7	8	18
31	40	7	12-13	21	87	159	7	12-13	32
32	42	8†	14-16	29	88	159	7	23-24	52
33	50	7	6-7	12	89	160	7	7-8	¶
34	50	7	5-6	10	90	160	7	8-9	73
35	57	7	8-9	12	91	160	7	8-9	¶
36	61	7	5-6	10	92	164	7	8-9	57
37	61	7	6-7	12	93	164	7	6-8	28
38	75	8	5-8	25	94	166	7	7-9	18
39	75	8	11-12	26	95	166	7	7-9	18
40	75	8	8-10	28	96	166	7	6-8	34
41	80	7	4-5	22	97	167	7	19-21	53
42	84	7	5-7	26	98	168	7	‖	68
43	84	7	5-7	40	99	168	7	12-14	¶
44	85	7	6	41	100	168	7	25-27	35
45	85	7	6	39	101	168	7	15-17	29
46	86	7	7	43	102	169	7	‖	35
47	86	7	7	48	103	169	7	‖	—
48	87	7	7	46	104	169	7	18-20	50
49	87	7	7	39	105	169	7	26-28	39
50	88	7	3-8	42	106	170	7	21-24	55
51	88	7	3-8	44	107	180	7	9-12	62
52	89	7	6	30	108	180	7	7-9	25
53	89	7	6	94	109	181	7	6-7	83
54	90	7	7-11	40	110	190	7	6-7	32
55	90	7	7-11	21	111	190	7	6-7	78
56	91	7	7-11	21	—	—	—	—	—

* In the above table each rat received the culture present in one tube, except Nos. 5, 6, and 13, which received each 4 cultures, and Nos. 10, 11 and 12, where 2 cultures were given each.

† This culture was grown for seven days at 25°, then placed for one day at 33° C.

‡ Blood survivals were present.

§ Death was not recorded.

‖ No infection occurred.

¶ These rats were bled for experiments on the fifteenth and sixteenth days.

shown in Table 2, gives a prompt infection, the parasites appearing in the blood in from four to seven days, and death results on about the fourth day after the trypanosomes have once been found. The short duration of the disease indicates that the organism is quite as virulent as that of the original blood.

After the tests with the second generation, none were made for some time, but were resumed with the thirty-second generation. The rat (Rat 30) inoculated with this culture gave the usually short period of incubation, but, unfortunately, a record of its death was not made. The serial tests made with cultures up to the seventy-fifth generation (Table 2) show, with the exception of Rats 31 and 32, that the duration of the infection from the time of inoculation (ten to twelve days) was not materially different from that produced by the second generation. The long survival (twenty-nine days) of Rat 32 must be ascribed to the exposure of the culture to 33 C. for one day. As shown by Novy and MacNeal, cultures at this temperature become non-virulent in two or more days. The course of the infection in Rat 31 was not due to this cause, but probably to a faulty inoculation.

After cultivation for one and a half years, that is, with the seventy-fifth generation, the duration of the infection became definitely prolonged, indicating a decided change in the infectivity of the trypanosomes. Rats 38, 39 and 40 inoculated at this time (Table 2) died on the twenty-fifth, twenty-sixth and twenty-eighth day, respectively. An inspection of Tables 2, 3 and 4 will show that the inoculation of seven-day cultures from the seventy-fifth to the one hundred and ninetieth generation, with very few exceptions, yields a chronic infection (Chart 1) which lasts more than twenty-five days, and in one instance was even prolonged to 126 days. The few instances of death in less than twenty days are often due to some other cause, since the number of trypanosomes in the blood, in such cases, is usually too small to produce a fatal result. In only four instances (Rats 98, 102, 103, Table 2; Rat 16, Table 3) did the rats fail to become infected. The uniformly positive results on inoculation has led us to believe that either these rats were accidentally not inoculated, or else the injections were made into the intestines. When Rat 16 (Table 3) was reinoculated sixty-three days later with a seven-day culture (Generation 100) it showed trypanosomes in its blood on the sixth day and died on the eighteenth day.

The average duration of the infection, consequent on inoculation of a seven-day culture, beginning with the seventy-fifth generation, was found for sixty-two rats listed in Table 2 (Rats 38 to 108, inclusive, less nine of uncertain duration), to be thirty-eight days, whereas the average with earlier generations for the twelve rats (Rats 25 to 37, Table 2) is but twelve and one-half days. This fact clearly shows that the culture has undergone considerable modification since the first year of cultivation, and it justifies the belief that in time the seven-day culture will become wholly avirulent for rats.

TABLE 3

SHOWING THE VIRULENCE OF CULTURES WHEN KEPT FOR 7, 14, 21 AND 28 DAYS

	Ages of Cultures											
	7 Days			14 Days			21 Days			28 Days		
Generation	No. of Rat	Period of Incubation, Days	Death, Days	No. of Rat	Period of Incubation, Days	Death, Days	No of Rat	Period of Incubation, Days	Death, Days	No. of Rat	Period of Incubation, Days	Death, Days
84	1	5-7	26	21	6	28	41	—	—	61	—	—
84	2	5-7	40	22	6-13	25	42	—	—	62	—	—
85	3	6	41	23	7	84	43	8-11	51	63	—	—
85	4	6	39	24	7-18	23	44	8-12	32	64	—	—
86	5	7	43	25	8-11	39	45	—	—	65	—	—
86	6	7	48	26	8-11	37	46	8-18	21	66	—	—
87	7	7	46	27	—	—	47	—	—	67	—	—
87	8	7	39	28	—	—	48	—	—	68	—	—
88	9	3-8	42	29	—	—	49	—	—	69	—	—
88	10	3-8	44	30	—	—	50	—	—	70	—	—
89	11	6	30	31	11-14	37	51	—	—	71	—	—
89	12	6	94	32	—	—	52	—	—	72	—	—
90	13	7-11	40	33	—	—	53	—	—	73	—	—
90	14	7-11	21	34	—	—	54	—	—	74	—	—
91	15	7-11	21	35	7-11	19	55	13-17	41	75	—	—
91	16	0*	—	36	4-7	29	56	—	—	76	—	—
92	17	4-7	42	37	7-13	30	57	—	—	77	—	—
92	18	4-7	24	38	4-7	11	58	6-8	32	78	—	—
93	19	4-7	13	39	—	—	59	—	—	79	—	—
93	20	4-7	12	40	—	—	60	11-14	265	80	—	—
Average		7.15	37.50		10-7	33		13.3	73.7			
Percentage of infection			100			55			30			0

* No infection.

The period of incubation, when a seven-day culture is used, does not vary as much as the duration of the infection. As a rule, daily examinations of the blood were not made. The rats were examined more often every two or three days, and so an exact period of incubation was not established. The average of the figures given in Tables 3

and 4 will be found to be about six days, though this is probably higher than it would be if daily examinations had been made. When this is compared with the five and four-tenths' day average inoculation of the five rats which received the second generation (Table 2), it will be seen that no material difference exists in this regard between the earliest and latest generations.

INFLUENCE OF AGE OF CULTURE ON INFECTIVITY

It has been pointed out in connection with Table 2 that the first generation of a culture, 15 to 40 days old, failed to infect rats. Novy and MacNeal[8] obtained similar results, and concluded that it was very probable that the cultures, when kept for three or four weeks at 25 C., lose their virulence. Inasmuch as no experiments had been made to test this point, it seemed desirable to ascertain just what influence age exerted on the pathogenicity of a culture. For this purpose a series of tests were made with ten consecutive generations, beginning with the eighty-fourth.

Since prolonged exposure of a culture to 25 C. causes considerable change in the medium and consequently in the form, and even destroys the life of the organism, it was found best to transfer the cultures, after they had been developed at 25 C. for seven days, to a cool room, the temperature of which, while not constant, was usually 10 to 15 C. Under these conditions the cultures showed little or no change during the first two weeks, but by the end of the third week, when actually 28 days old, considerable alteration of form was noted. A goodly number of the organisms, however, were still actively motile, and when transplanted to a fresh medium invariably gave a growth, which, though slight, on further transplantation yielded typical rich cultures and these infected rats the same as the original seven-day culture. It may be noted, in this connection, that the 28-day-old culture, while transplantable, was without effect on animals (Table 3).

At the end of seven, fourteen, twenty-one and twenty-eight days, after inoculation, the cultures were tested on rats in the usual way. The rats were injected in duplicate, each receiving the contents of one tube. The results of this series of tests are summarized in Table 3. It will be seen from this table that the cultures, when grown for seven days at 25 C., produced infection in nineteen of the twenty rats tested, the single exception being that of Rat 16. The possible reason

8. *Jour. Amer. Med. Assn.*, 1903, 41, p. 1266; *Jour. Infect. Dis.*, 1904, 1, p. 1.

for this failure has already been given, and it is safe to conclude, in the light of all the other positive results obtained with seven-day cultures (Table 2), that a culture at this stage of its growth is invariably infective to rats.

On the other hand, the fourteen-day cultures (seven days at 25 C., and seven days at room temperature) were found to infect only eleven out of twenty rats, that is, 55 per cent. The twenty-one-day cultures were even less infective, only six out of twenty (33 per cent.) being positive, whereas the twenty-eight-day cultures were uniformly non-virulent. It will be seen, therefore that the increase in age of the

TABLE 4

SHOWING THE VIRULENCE OF CULTURES WHEN KEPT FOR 7, 14 AND 21 DAYS

	Ages of Cultures								
	7 Days			14 Days			21 Days		
Generation	No. of Rat	Period of Incubation, Days	Death, Days	No. of Rat	Period of Incubation, Days	Death, Days	No. of Rat	Period of Incubation, Days,	Death, Days,
136	1	2-6	31	11	—*	—	21	—	—
136	2	2-6	53	12	—	—	22	—	—
137	3	2-6	40	13	—	—	23	—	—
137	4	2-6	28	14	—	—	24	—	—
138	5	2-6	31	15	6-9	39	25	—	—
138	6	2-6	64	16	6-9	64	26	—	—
139	7	6	30	17	2-6	60	27	—	—
139	8	6	27	18	2-6	46	28	—	—
140	9	6	30	19	2-9	12	29	—	—
140	10	6	30	20	2-9	33	30	—	—
Average		6	36.4		8	42.3			
Percentage of infection			100			60			0

* No infection, consequently no death occurred.

cultures (Generation 84-93) is accompanied by a rapid loss of virulence, the infection dropping from 100 per cent. at the end of seven days to 55 per cent. in fourteen days, 33 per cent. in twenty-one days, to *nil* per cent. in twenty-eight days.

After an interval of one year the cultures were again tested and the results brought together in Table 4. Since the twenty-eight-day cultures in the previous trial had been found to be non-infective, no tests were made with tubes of this age. The cultures were now in the one hundred and thirty-sixth to one hundred and fortieth generation, and

were, therefore, nearly three years out of the animal body. As before, the seven-day culture proved to be infective for all the rats (ten) tested, or 100 per cent.; the fourteen-day culture infected six out of ten rats, or 60 per cent., thus showing practically no change. The twenty-one-day culture, however, failed to infect, thereby indicating a change in the organisms as compared with the previous year.

It would not be safe to conclude from the foregoing that the twenty-one-day cultures were always devoid of pathogenicity. Thus, when rats were given from five to ten bi-weekly injections of such a culture, with the object of immunization, five out of twenty-one (23 per cent.)

TABLE 5

SHOWING EFFECT OF MULTIPLE INJECTIONS OF 21 DAY OLD CULTURES

No. of Rats	Generation	No. Injections	Period of Incubation Days	Death, Days	Remarks
1	144-147	6	—	—	No infection
2	144-147	6	—	—	No infection
3	144-147	6	—	—	No infection
4	144-147	6	—	—	No infection
5	144-147	6	—	—	No infection
6	144-147	6	—	—	No infection
7	144-146	5	—	16	No infection
8	144-147	6	—	—	No infection
9	144-146	5	3-18	41	.....
10	144-147	6	—	—	No infection
11	144-146	5	4-15	53	.....
12	144-146	5	10-15	46	.....
13	144-146	5	12	48	.....
14	144-146	5	—	15	No infection
15	144-148	9	—	32	No infection
16	144-148	9	30	142	.....
17	144-149	10	—	—	No infection
18	144-149	10	—	—	No infection
19	144-149	10	—	—	No infection
20	146-151	10	—	—	No infection
21	146-151	10	—	—	No infection

The period of incubation is not definitely determined; the figures given represent the last negative and first positive examination.

became infected (Table 5). It would seem, therefore, that variations in the conditions of cultivation, such as slight differences in the medium or in the rate of multiplication, influence appreciably the pathogenicity of the twenty-one-day cultures. It is quite possible that in a given tube a culture may rapidly develop and as a result undergo degeneration more readily and thus become non-infective, while a companion tube with less growth may contain virulent organisms. This would seem to be the reason why frequently only one or two rats became infected when inoculated with apparently the same material. The set of twenty-

one-day rats which received multiple inoculation were given identically the same cultures, and yet only 23 per cent. developed trypanosomes. This variation can hardly be explained as due to individual susceptibility of the rats.

The period of incubation, after injection of a culture, was not determined as accurately as perhaps it should have been. It is to be regretted that daily examinations of the blood for trypanosomes were not made, but this was quite impossible owing to the large number of animals which required attention. As shown in Tables 2 and 4, the rats which received the seven-day culture usually showed trypanosomes on the sixth or seventh day, the average period of incubation in the former table being seven and in the latter six days. Had daily examinations been made, it is not unlikely that the average would have been reduced to nearly five days. The average period of incubation, when the fourteen-day culture was used, is apparently longer, for in Table 3 it comes to ten days, while in Table 4 it is eight days. In the case of the twenty-one-day culture the average is increased to thirteen days. It is rather to be expected that with the progressive weakening of the culture, the incubation period should be prolonged.

The duration of the disease, as expressed in Tables 2-4, refers to the number of days which elapsed between the date of inoculation and of death. It would seem as if this could be given with accuracy, but such is not quite the case, for the reason that, not infrequently, the test animal succumbs to an intervening disease. Thus Rats 19, 20 and 38 (Table 3) died in thirteen, twelve and eleven days, respectively. Neither at the time of death nor at any previous day did they show more than a few parasites, and this small number could not account for the rapidly fatal outcome. Exceptionally, when the animal is in an enfeebled condition, trypanosomes may appear in large numbers (about 100 per field) on about the fourteenth day, and death may be due to this cause. In general, however, the infection persists for several weeks, at times for two or three months; and in one instance (Rat 60, Table 3) it lasted for 265 days.

The average duration for rats inoculated with the seven-day culture is 37.5 days (Table 3) and 36.4 days (Table 4); for those which received the fourteen-day culture the averages are 33 and 42.3 days, respectively; while for those which were given the twenty-one-day culture the average is 73.7 days. This latter high average is due to the extremely long survival of Rat 60, and if this is excluded the average drops to 35.4 days.

It will be seen, therefore that the average duration of the infection in rats produced by the trypanosoma brucei when cultivated for two or three years is approximately the same, although considerable individual variation, as seen in the tables, does exist.

EFFECT OF SMALL DOSES OF CULTURE

It will be seen from the foregoing results that one tube of a seven-day culture, even in the latest generations, invariably infects the white rat, and that such cultures when kept for an additional period of from one, two or three weeks, progressively lose their infecting power. If the latter fact was merely due to a decrease in the number of organisms, then a somewhat similar result could be expected by injecting fractional doses of a single culture.

With the object of determining the least infective dose, a seven-day culture was taken up in sterile salt solution (0.85 per cent.) and diluted in series so as to yield 1:10, 1:100, 1:1,000, etc., suspensions. When 1 c.c. of these suspensions was injected intraperitoneally into each of two rats, it was found that the 1:1,000 suspension uniformly failed to infect. Thus, eighteen rats, in sets of two, received such suspensions from nine different cultures, and in none was an infection obtained.

The tests made with the 1:10 and 1:100 dilutions gave frequent, though by no means constant infections. In the early summer of 1912 eighteen rats, most of which had received previous injections of fourteen-, twenty-one- and twenty-eight-day cultures, were tested for a possible immunity, each receiving one-tenth of a culture tube, and every one became infected. A few months later, in October, four consecutive tests, made on eight clean rats, gave a negative result with the one-tenth-culture dose, and it seemed therefore as if the organism had become considerably less virulent during the few intervening months. Further tests, however, showed that this avirulence was merely temporary, due to some as yet unrecognized cause.

In endeavoring to find the cause of this temporary loss of virulence of the one-tenth-culture dose, attention was first drawn to a variation in the procedure of making the subcultures. It was found that the non-infective cultures were prepared by transferring the growth, by means of a pipet, from one tube to each of two fresh blood-agar tubes, whereas in the case of the infecting cultures, the inoculation was made into four or five fresh tubes. It was conceivable, therefore, that the

former received a heavier inoculation, and consequently developed an earlier maximum growth than the latter. As a result of the more rapid growth, an earlier degeneration could be expected, in which case the culture, though but seven days old when used, would be less infective than the more slowly developing cultures. To test this hypothesis, two series of cultures reproducing the above conditions were carried through four generations, and then tested on rats. These cultures (Generation 140) at the end of seven days were equally rich and showed no difference in the appearance of the organisms. The results, given in Table 6, likewise show no difference between the two series as regards the effects on rats. The early death of five out of eight rats was due to an intervening infection. Hence some other explanation had to be sought.

TABLE 6*

SHOWING THE EFFECT ON VIRULENCE OF THE QUANTITY OF CULTURE TRANSPLANTED

Transplants Made from 1 to 2 Tubes				Transplants Made from 1 to 4 Tubes			
No. of Rat	Amount Injected	Period of Incubation, Days	Death, Days	No. of Rat	Amount Injected	Period of Incubation, Days	Death, Days
1	0.1	4-6	28	5	0.1	6-8	43
2	0.1	4-6	7	6	0.1	8-10	14
3	0.01	8-10	43	7	0.01	8-10	14
4	0.01	..	10	8	0.01	10-13	14

* Generation 140 was used in this experiment.

Another explanation of the variable results following the injection of one-tenth of a culture was sought in the temperature of the salt solution employed in the preparation of the suspensions. It seemed likely that suspensions made with a moderately warm solution would be less active or even avirulent as compared with those prepared at a lower temperature. Accordingly, a series of comparative tests were made to determine this point. In one set, after the seven-day culture was cooled in ice water, the growth was taken up in iced salt solution (0.85 per cent.) and the desired suspensions were prepared with the chilled solution. Even the syringes employed were first rinsed in the cold, sterile water. For the other set the suspensions were prepared with a salt solution previously warmed to 30 C.

The results obtained in two such tests (Table 7) were so markedly opposed as to make it clear that the temperature of the suspension was not a factor.

A third attempt to account for the above-mentioned results gave an equally unsatisfactory answer. It was suggested that the cultures which usually were kept on the floor of the hot room were possibly exposed to a varying temperature, and that a rise of a few degrees might render the culture more virulent. To bring out the effect of temperature, accordingly a series of tests were made, with cultures grown for seven and nine days, respectively, at 25 to 27 C., and compared with similar tests of cultures grown at 30 C. The nine-day cultures at 30 C., while very rich, showed a considerable number of rounded-up organisms, which, however, were still actively motile, whereas those grown for a like period at 25 to 27 C. contained perfectly normal flagellates.

TABLE 7

SHOWING RESULTS OF INOCULATION WITH SUSPENSIONS MADE AT DIFFERENT TEMPERATURES

Cultures Taken Up in Water at									
0 C.					30 C.				
Generation	No. of Rat	Amount Injected, Tube	Period of Incubation, Days	Death, Days	Generation	No. of Rat	Amount Injected, Tube	Period of Incubation, Days	Death, Days
149	1	0.1	†	—	149	5	0.1	6-8	38
149	2	0.1	†	35	149	6	0.1	10-12	26
149	3	0.01	†	16	149	7	0.01	6-8	26
149	4	0.01	†	—	149	8	0.01	†	—
150	9	0.1	15-18	39	150	13	0.1	†	—
150	10	0.1	8-12	29	150	14	0.1	†	—
150	11	0.01	†	—	150	15	0.01	†	—
150	12	0.01	†	—	150	16	0.01	†	—

† No infection occurred.
Where no death is recorded, the rats lived for more than 5 months uninfected.

On examination of Table 8 it will be seen that the injection of one-tenth of a culture produced an infection in fifteen out of twenty-four rats, while that of one-hundredth of a culture gave a positive result in only eight, or one-third of the rats tested. The death of a number of the rats within two weeks after inoculation was due to a troublesome intercurrent infection, and undoubtedly affected the total result. On the whole, however, it is safe to conclude that neither the temperature nor duration of cultivation influenced the virulence of the culture.

Therefore, the occasional failure of a fraction of a culture to infect is not due to any one of the three factors discussed above, neither can it be due to a variation in the medium, since the same nutrient agar was employed in all tests. Only one explanation seems to remain,

TABLE 8

SHOWING THE EFFECT OF TIME AND TEMPERATURE ON THE VIRULENCE OF CULTURES

	Cultures Grown at 25-27 C. for								Cultures Grown at 30 C. for							
	7 Days				9 Days				7 Days				9 Days			
	No. of Rat	Amount Injected, Tube	Period of Incubation, Days	Death, Days	No. of Rat	Amount Injected, Tube	Period of Incubation, Days	Death, Days	No. of Rat	Amount Injected, Tube	Period of Incubation, Days	Death, Days	No. of Rat	Amount Injected, Tube	Period of Incubation, Days	Death, Days
Exp. 1																
Gen-	1	0.1	8-10	41	5	0.1	6-8	13	9	0.1	8-10	69	13	0.1	10-12	43
era-	2	0.1	8-10	58	6	0.1	†	7	10	0.1	12-14	59	14	0.1	8-10	42
tion	3	0.01	†	—	7	0.01	6-8	*	11	0.01	†	26	15	0.01	10-12	*
152	4	0.01	†	—	8	0.01	†	—	12	0.01	14-16	*	16	0.01	6-8	*
Exp. 2																
Gen-	17	0.1	5-7	22	21	0.1	†	9	25	0.1	3-5	52	29	0.1	11-13	*
era-	18	0.1	5-7	33	22	0.1	†	8	26	0.1	7-9	36	30	0.1	7-9	25
tion	19	0.01	11-13	60	23	0.01	7-9	36	27	0.01	†	14	31	0.01	†	12
153	20	0.01	5-7	64	24	0.01	11-13	20	28	0.01	†	6	32	0.01	†	—
Exp. 3																
Gen-	33	0.1	†	19	37	0.1	†	—	41	0.1	†	—	45	0.1	8-10	50
era-	34	0.1	9-11	17	38	0.1	†	9	42	0.1	†	23	46	0.1	†	10
tion	35	0.01	†	10	39	0.01	†	—	43	0.01	†	10	47	0.01	†	9
153	36	0.01	†	—	40	0.01	†	5	44	0.01	†	—	48	0.01	†	5

*Death was not recorded. †See foot-notes to Table 7.

namely, a variation in the number of trypanosomes injected. Previous to making up a suspension the cultures were always examined, and it is possible that in some instances an unusually rich mass of flagellates was thus removed from the tube, thus decreasing the number actually injected. The injection of a definite number of cultural trypanosomes, ascertained by direct count, would probably throw light on this question.

INOCULATION OF MICE

As expected mice were found to be very susceptible. The period of incubation, following the intraperitoneal injection of a seven day culture, was about the same as in rats, and varied somewhat with the amount injected. Thus, as shown in Table 9, it was about four to six

TABLE 9

SHOWING THE RESULTS OF INOCULATION OF MICE WITH CULTURES

Generation	No. of Mice	Amount Injected Tube	Period of Inoculation, Days	Death, Days
143	1	1	5-6	18
143	2	1	†	2
143	3	0.1	†	—
143	4	0.1	5-6	13
143	5	0.01	7-8	17
143	6	0.01	†	
144	7	1	4-6	9
144	8	1	4-6	15
144	9	0.1	6-8	12
144	10	0.1	6-8	23
144	11	0.01	8-10	14
144	12	0.01	8-10	12
145	13	0.1	6-8	14
145	14	0.1	6-8	14
145	15	0.01	8-12	14
145	16	0.01	8-12	14
145	17	0.001	8-12	*
145	18	0.001	8-12	13

* Death was not recorded. † See foot-notes Table 7.

days when an entire culture was injected; with one-tenth and one-hundredth of a culture it was prolonged to six to eight and eight to ten days, respectively. As will be seen, even one-thousandth of a culture is capable of producing an infection. One of the mice which received this small dose (Mouse 17) showed trypanosomes in its blood on one day, and although examined repeatedly during the next few months they were not found again. While this would seem to be a recovery, it is more probable that an error in observation was made. In the routine examination of a large number of animals it has been

customary to make eight to ten or more fresh blood preparations on a slide, and then examine these in regular order. Nothwithstanding the care taken to avoid mistakes in making such serial examination they occurred at times. The duration of the disease, from the time of inoculation, was less than observed with the rats; averaging fourteen and four-tenths days with nine days as the minimum, and twenty-three days as the maximum.

INOCULATION OF DOGS

Only a limited number of tests with dogs were made, and these sufficed to show that the cultures, approximately three years under cultivation, were infective. The injection was made, as usual, intraperitoneally. Dog 1 received five injections, each of ten tubes, in the course of sixteen days. Parasites appeared in its blood on the

TABLE 10
SHOWING THE RESULTS OF INOCULATION OF CULTURES INTO DOGS

No. of Dog	Weight, gm.	Date of Inoculation	Generation	Amount Injected, Tubes	Period of Incubation, Days	Death, Days
1	8700	Dec. 24, 1912	140 141 142	5x10	13-18	44
2	2950	Jan. 28, 1913	144	10	8	127
3	2900	Feb. 18, 1913	147	1	7-10	19
4	2600	Feb. 18, 1913	147	1/10	No infection	39

eighteenth day, rapidly increased in numbers, and death occurred in forty-four days. Dog 4, which received only one-tenth of a culture, although examined twice a week, failed to show trypanosomes and died on the thirty-ninth day. Dog 3 after receiving an injection of a single culture showed one trypanosome on the tenth day, after which the number rose to ten per field or more. The animal became very sick and died of acute infection on the nineteenth day. On the other hand, in Dog 2, which received an injection of ten cultures, the infection pursued a very chronic course. The parasites appeared on the eighth day and continued to be present, with frequent intermissions, up to the time of death (127 days). The number of trypanosomes was usually only one or two per field, but once it rose to ten per field.

INOCULATION OF RABBITS

The rabbit, when inoculated with the virulent trypanosoma brucei, presents a sub-acute infection characterized usually by a period of incubation of two to six or eight days, though it may be considerably

longer (even thirty-one and forty-nine days) depending on the amount of the virus injected. The duration of the disease is from ten to fifty days,[9] but with minute doses this may be greatly prolonged.

In view of the fact that the rabbit possesses a fair degree of natural resistance no tests of the infectivity of the culture were made with small amounts of such material. Instead the rabbits in one set were given an intraperitoneal injection of the contents of ten cultures. As shown in Table 11, the rabbits thus injected were found, with one exception, to resist this large inoculation. Rabbit 5 died in thirteen days, but not from trypanosome infection. The other animals,

TABLE 11

SHOWING THE EFFECT OF SINGLE AND MULTIPLE INJECTIONS OF CULTURES INTO RABBITS

No. of Rabbit	Generation of Culture	Date of Injection	Amount Injected, Tubes	Period of Incubation Days	Death, Days	Remarks
1	144	Jan. 28, 1913	10	†		Used for an immunity test on the ninety-eighth day
2	145	Jan. 31, 1913	10	†		—ditto on the ninety-fifth day
3	145	Jan. 31, 1913	10	†		—ditto on the one-hundred and twenty-second day
4	145	Feb. 11, 1913	10	†		—ditto on the one-hundred and eleventh day
5	146	Feb. 11, 1913	10	†	13	
6	147	Feb. 14, 1913	10	†		—ditto on the two-hundred and third day
7	148	Feb. 15, 1913	10	66-70		Alive Jan. 1, 1914 (308 days)
8	145-149	Jan. 31-Mar. 4	10x10	†		Used for immunity test on the ninety-fifth day
9	145-149	Jan. 31-Mar. 4	10x10	17-49	58	
10	145-147	Jan. 31-Feb. 4	4x10	1-12		Alive July 3, 1914 (518 days)
11	146-151	Feb. 11-Mar. 14	10x10	†		Used for immunity test on the one-hundred and tenth day
12	146-151	Feb. 11-Mar. 14	10x10	†		—ditto on the two-hundred and fifth day
13	146-151	Feb. 11-Mar. 14	10x10	†		—ditto on the two-hundred and fifth day

† No infection occurred.

although examined at least twice a week, failed to show any organisms except in the case of Rabbit 7, in the blood of which a single trypanosome was found on the seventieth day. About 5 drops of blood from the ear of this rabbit were injected into each of two rats. Trypanosomes appeared in the blood of these rats on the fifth day. Ten days later another parasite was detected.

At the same time as the above, six rabbits (Rabbits 8 to 13) were given bi-weekly injections, each of ten tubes, intraperitoneally. Rabbit 10 showed trypanosomes (one per field) on February 12, the twelfth

9. Laveran and Mesnil, Trypanosomes et Trypanosomiases, 1912, p. 440.

day after the first inoculation. Two days later they were slightly increased (four per field), after which they disappeared and were not found again until March 11, 21 and 28, when a single trypanosome was detected on each of those days. Examinations made during the months of April and May did not reveal the presence of the parasite. On June 18 one organism was found; alopecia, ulceration of genitalia, and of nose were noted. After July and August, during which no organisms were seen, further examinations were omitted. At present (July 3, 1914) the animal is in excellent condition, showing no signs of lesions.

Rabbit 9 showed a single trypanosome on March 21, the seventeenth day following the last or tenth injection, (forty-ninth day from the date of the first injection). It was negative on March 25, but on March 28 the trypanosomes were ten per field. It died two days later, the fifty-eighth day from the first injection. The other four rabbits which received multiple injections, showed no indication of an infection until the time when they were used for immunity tests.

It will be seen from these experiments with rabbits that at the end of three years of cultivation, the trypanosoma brucei has suffered a marked loss of pathogenicity. Owing to the richness of the material injected the occasional infection must be ascribed to individual susceptibility.

INOCULATION OF GUINEA-PIGS

The inoculation of the virulent trypanosoma brucei into guinea-pigs gives rise, as in the case of rabbits, to a subacute or even chronic infection having a period of incubation of two to four days, and of variable duration of fifteen to thirty days,[10] although much longer survivals are known. In view of this fact it is to be expected that the injection of a culture, particularly when attenuated under the artificial conditions of cultivation, should be followed by a strictly chronic infection or none at all. The results of numerous tests show that this actually does happen, and that at the end of three years' cultivation the organism is practically avirulent for the guinea-pig.

It had been shown in this laboratory that the injection of early generations of trypanosoma brucei infected guinea-pigs. In our own work we found this to hold true for the fortieth generation, a single culture of which, as shown in Table 13, produced a fatal though somewhat prolonged infection.

10. Laveran and Mesnil, p. 441.

No further test was made with cultures until the one hundred and fifth generation was reached. It was then found that a single culture, given intraperitoneally, failed to infect, and this fact was soon confirmed by a series of similar inoculations. Twenty-five guinea-pigs were injected with the one hundred and fifth to the one hundred and forty-fifth generation, inclusive, each with the culture present in a single tube. Of these, four died within the first week, two in the

TABLE 12

SHOWING NEGATIVE RESULTS AFTER INOCULATION OF GUINEA-PIGS WITH ONE CULTURE.

Generation	No. of Guinea-Pigs Inoculated	Results
105	2	One died on the thirteenth, the other on the twenty-fifth day; both negative
109	2	Examined for 79 days, negative
116	2	Examined for 36 days, negative
131	1	Died on sixteenth day, negative
132	2	One died on twentieth day, other examined for 114 days, both negative
133	1	Died on sixteenth day, negative
134	3	Examined for 130, 136, 154 days, respectively; all negative
135	1	Examined for 156 days, negative
136	1	Died on forty-first day, negative
142	2	Examined for 41, and 95 days, respectively, negative
145	4	One died on the eighth day, another on eighteenth day; the others were examined for 46, 52 days, respectively, negative

TABLE 13

SUMMARY OF POSITIVE RESULTS OBTAINED IN GUINEA-PIGS BY INOCULATION OF CULTURES

No. of Guinea-Pig	Generation	No. of Tubes Injected	Period of Incubation, Days	Death, Days
16.94	40	1	14-15	59
4.190	140	10	39-40	50
5.190	140	10	21-24	210
5.147	146	10	14-18	53
6.147	146	10	28-32	158
4.68	133-139	10x1	51-92	268
5.105	136-144	10x10	7-70	104

second week, four in the third week and one in the fourth week, without showing any trypanosomes. The remaining fourteen animals were under observation for thirty-six to 156 days, after which they were used for immunity tests. At no time could parasites be detected in their blood. It is clear, therefore, that this strain had become attenuated to such an extent that a dose of a single culture was unable to infect.

A summary of these tests, including the four animals that died in the first week is given in Table 12.

Inoculation with Ten Cultures.—Inasmuch as it was evident that a single culture would not infect, it was desirable to test the effect of a larger dose. For this purpose the growth present in ten culture tubes was taken up in the usual way and injected. This was made intraperitoneally except in four guinea-pigs, where the injection was subcutaneous. The examinations of the blood were made twice a week at first, and later on alternate days. The animals were thus kept under observation as long as they lived or until used for immunity tests two to five months later.

Of 72 animals thus inoculated with Generations 133 to 153, inclusive, 5 died within fourteen days and were negative. Of the remaining 67 animals, 2 died during the second, and 7 during the third fortnight, all being negative. Of these 67 animals, 4 became infected after a period of incubation of eighteen, twenty-four, thirty-two and forty days, respectively. In other words, 1 out of every 17 guinea-pigs which survived the first fortnight became infected. It is evident therefore, that the culture when given in large doses is not wholly avirulent for these animals.

This result may be due to particularly rich cultures which happened to be used in those instances, or it may be ascribed to individual susceptibility of the animals. An effort was made to ascertain the cause of this occasional infectiveness by growing the cultures in parallel sets at 25 and 30 C. for seven and nine days, at the same time that similar tests were made with rats (Table 8). Although thirty-four animals were thus tested not a single infection was induced. Likewise tests to ascertain the effect of the temperature of the salt solution which was used to make up the suspension, employing this either ice-cold or at 30 C. failed to show any difference, and the animals, fifteen in number, did not become infected. On the supposition that in the first two positive cases mentioned some of the cultures may have been introduced subcutaneously by accident and that injection by this latter route might be a factor, it was deemed best to make some comparative trials. In one such test two guinea-pigs received the usual intraperitoneal injections, while two others were given subcutaneously a like dose from cultures grown side by side with those used for the former. The latter failed to become infected, while both of the animals which received the intraperitoneal injection became positive.

It is worthy of note that the four positive infections occurred in sets of two, and this fact would offset the explanation of individual susceptibility. The first set received Generation 140 and the second set Generation 146. In view of the failure to trace these results to any definite cause it is reasonable to suppose that some special quality of the blood agar medium, very accidental in its occurrence, influenced the cultures and was responsible for the result. That the result was not due to a mere numerical richness of the culture is seen from the following tests:

Inoculation with One Hundred Cultures.—If the occasional infection of guinea-pigs with a culture were due to a special richness of the material injected it would seem that a much larger dose than that of ten tubes should produce positive results. Accordingly, it was decided to test the effect of one hundred such cultures on each of several animals. The growth from one hundred tubes, which examination showed to be extremely rich, was taken up in 20 c.c. of salt solution, and this suspension was injected intraperitoneally into a guinea-pig. Six animals were given this dose of one hundred cultures. The first and second pairs received Generation 141, the third pair Generation 142. Two of these died within a week and were negative; the remaining four were examined at first daily and later on alternate days for seventy-eight to eighty-four days without revealing any trypanosomes. It is seen, therefore, that while Generation 140 infected two guinea-pigs in a dose of ten tubes, in this case Generations 141 and 142 failed to produce a like result, although the amount injected was tenfold.

Multiple Injections of One Culture.—As a further test of the action of cultures, and especially with the object of securing material for immunity tests, a series of thirteen guinea-pigs were inoculated, either weekly or bi-weekly, each time with the growth of a single culture tube. Six of these died a day or two after having received two, three, four, four, eight and eight injections, respectively, representing an interval of eight to thirty-seven days after the first injection. In these the usual examinations were made with negative results.

Seven of the above set were carried through the tenth and last injection. One died on the sixty-ninth day, and five were used for immunity tests on the ninety-eighth to the one hundred and third day after the first inoculation. At no time did these six show any sign of trypanosomal infection. One of the set, however (Guinea-Pig 4.68, Table 13). which received in the course of forty-one days ten injections of Generations 133 to 139, inclusive, showed a very few trypanosomes on the

fifty-first day after the last injection (ninety-two days after the first). The period of incubation in this case was therefore at least fifty-one days; it may have been longer since obviously it could not be determined which one of the ten injections actually infected. It is interesting to note that although much of the same culture material was used in the other animals, only this one became infected. The course of the infection in this particular guinea-pig will be discussed later.

Multiple Injections of Ten Cultures.—At about the same time as the above, each of another set of twelve guinea-pigs was given bi-weekly injections of ten cultures. Two of the animals died after receiving two and three injections respectively. The remaining ten guinea-pigs were given ten injections each. Of these, two died on the fourteenth and twenty-ninth days after receiving the last injection (45 and 61 days after the first), the examinations of the blood being negative. Seven of the remaining gave likewise negative results on frequent, two or more, weekly examinations, and were therefore used for immunity tests after 30, 42, 55, 55, 70, 70 and 91 days elapsed from the time of the last injections, or 61 to 122 days from the first. One guinea-pig in this set (Guinea-Pig 5.105, Table 13) showed a very slight infection on the seventh day following the last injection (70 days after the first). The period of incubation, as in the other infection with multiple injections (Guinea-Pig 4.68) obviously could not be determined. The course of the infection, which was very slight, will be described later.

Course of the Infection.—The several positive infections on inoculation of cultures which have been referred to in the preceding text are summarized in Table 13, and it will be pertinent to briefly outline the course of the disease, as indicated by the trypanosome contents of the blood in these animals.

Guinea-pig 16.94 received an injection of 1 culture, Generation 40, on January 4, 1911. Daily examinations were made and trypanosomes found in very scanty numbers (0.05 per field) on January 19. After that date the examinations were made two or three times a week until death, which occurred on the fifty-ninth day. The infection was characterized by the continuous presence of trypanosomes in the blood, as seen from the following:

Jan. 14	Inoculated	Feb. 7	5 per field
" 19	0.05 per field	" 9	35 " "
" 22	0.5 " "	" 14	50 " "
" 24	0.05 " "	" 17	7 " "
" 26	1 " "	" 20	½ " "
" 28	3 " "	" 25	2 " "
Feb. 2	3 " "	March 1	50 " "
" 4	4 " "	" 4	died, 59th day.

This constant presence of parasites, their gradual rise in number to a maximum, then a remission followed by a second rise shortly before death are features fairly characteristic of infection with the virulent trypanosoma brucei. Two years later, in only one of the other positive infections was a like course observed, and hence this can be next considered.

Guinea-pig 4.190 received an injection of 10 cultures, Generation 140, on Dec. 24, 1912. Through oversight examinations were made only once or twice weekly during the first four weeks (seven tests); after that, however, they were made daily and parasites were found, after eleven consecutive negative examinations, on February 2. They continued present, as shown in the subjoined tabulation, until death, which occurred ten days later. The rapid course of the infection in this animal is in striking contrast with that of its mate, Guinea-pig 5.190, which received a like injection on the same day.

Dec. 24	Inoculated	Feb. 6	5 per field
" 2	1 per field	" 8	25 " "
" 3	1 " "	" 9	10 " "
" 4	5 " "	" 11	50 " "
" 5	4 " "	" 12	died 50th day.

Guinea-pig 5.147 was given an injection of ten cultures, Generation 146, on February 7. Five negative examinations were made during the first fortnight. Parasites were found on February 25, and in increased numbers on the day following; they then disappeared, as shown by negative findings in the next seven examinations. On March 10 and 12 they reappeared in small numbers, but were again absent in the next five tests. On March 24 they appeared for the third time, followed by a considerable increase on the 26th, but on the 28th they were again absent and remained so until death, which occurred on April 1 (fifty-three days).

Feb. 7	Inoculated	Mch. 12	½ per field
" 25	1 per field	" 24	5 " "
" 26	10 " "	" 26	75 " "
Mch. 10	½ " "	April 1	died, 53d day.

The noteworthy feature of the infection in this animal is the occurrence of three remissions, each being characterized by an apparently complete disappearance. Similar intermissions were observed in the remaining guinea-pigs. No sub-inoculation of the blood during these intermissions was made.

Guinea-pig 6.147, the mate of the preceding, received on the same day a like dose. Nine examinations during the following four weeks were negative. Trypanosomes appeared on March 11, were present the next day, and then disappeared. After six negative examinations, on alternate days, they were again found on March 26 and 28, and April 1. Absent on the 8th, they reappeared on the 11th, and again disappeared and remained undetected in the nine following examinations. On May 23 they were again found, but not on the 27th and 30th. The parasites were met with on June 3d and 6th; they were not found in the next ten examinations, which were made between June 10 and July 12.

Feb. 7	Inoculated	April 4	1 per field
March 11	½ per field	" 11	20 " "
" 12	½ " "	May 23	2 found
" 26	2 found	June 3	½ per field
" 28	10 per field	" 6	2 " "
		July 15	died, 158 days.

Guinea-pig 5.190, the mate of one of the preceding, was given an injection of ten cultures on Dec. 24, 1912. After four negative examinations, trypanosomes were found on January 17. Unlike the rapid course of the disease in its mate, the infection in this animal took on a very chronic course characterized by the frequent presence of parasites, although these occurred only in scanty numbers, as seen from the observations noted below.

Dec. 24	Inoculated	Feb. 18	3 found
Jan. 17	1/20 per field	Feb. 19	1 per field
Jan. 18	1 " "	Feb. 20	1 " "
Jan. 19	5 " "	Feb. 21	1 " "
Jan. 20	1 " "	March 22	1 found
Jan. 21	1/5 " "	April 8	5 per field
Jan. 23	1/10 " "	April 11	5 " "
Jan. 24	1/2 " "	April 25	1 " "
Feb. 2	1/2 " "	May 6	10 " "
Feb. 3	1/2 " "	May 13	2 " "
Feb. 5	1/2 " "	May 16	1 " "
Feb. 6	1/2 " "	May 30	1 " "
Feb. 8	1 " "	June 13	5 " "
Feb. 9	1 " "	July 1	2 " "
Feb. 12	2 found	July 12	3 " "
Feb. 13	1 "	July 15	10 " "
Feb. 16	2 "	July 22	died, 210 days.

The intermittent presence of trypanosomes, in relatively small numbers, for a long period of time is a marked feature of the infection. The first intermission occurred after January 24, when eight consecutive daily examinations were negative. The second intermission occurred after February 21, when eighteen consecutive examinations failed to reveal the parasite. After March 22, when a single trypanosome was noted, five following examinations were negative. Of four examinations made in the two weeks following April 11, only the last was positive. In the next three weeks, negative findings were made on April 29, May 2 and 9. The organism was seen on the thirteenth and sixteenth, when again a period of remission followed on the twentieth, twenty-third and twenty-seventh of May. On the thirtieth the parasite was found in small numbers (one per field). Ten negative and four positive examinations were made between May 30 and July 15. The number of trypanosomes seen was never more than ten per field. Death occurred on the two hundred and tenth day after the first injection.

A few drops of the ear blood of this animal were injected on January 17 into a white rat and a guinea-pig. Two days later a similar injection was made into another rat. Parasites appeared in the blood of the first rat on the third day, after which they rapidly increased in numbers, reaching 200 per field on February

4. Death occurred on February 11, the twenty-fifth day. In the second rat, a similar rapid infection was noted. The parasites appeared in the blood on the second day, and rose to 200 per field before death, which took place on February 6, the eighteenth day.

In striking contrast, however, the guinea-pig which was inoculated on January 17 failed to show any organism in the first twelve examinations. Parasites appeared on the eighteenth day, February 4, about one-half per field. Six examinations between this date and March 4 were negative. On that date, trypanosomes were present, twenty-five per field. The blood was searched on the 7th, 11th, 14th and 18th, with negative results. The parasites again appeared on March 21, about fifty per field, but four days later they were again absent, and the blood remained free of organisms until May 27, although eighteen examinations were made. The animal was therefore apparently free from organisms for more than two months. Three days later the organisms disappeared and were again absent for a period of eighty-seven days, although nineteen examinations were made, when positive, two trypanosomes were seen; four days later, three were found. August 29, or 224 days from the time of its injection, was the last record of examination. No note of its death was made. On May 26, as a test for the possible presence of trypanosomes, some of the ear-blood of the animal was injected into a rat. Parasites appeared on the thirteenth day (June 8), after which they rapidly increased to 100 per field and then slowly decreased in numbers until after a period of thirty-four days, when they disappeared; in the next seven examinations, which terminated August 28, the animal revealed no parasites. Further examinations and a record of its death were not made.

Guinea-pig 4.68, which received ten injections, each of one culture, in the interval between November 6 and December 17, was the only one of seven similarly treated animals which became infected. Although examined twice a week, it showed no parasites until February 6 (fifty-one days from the last injection), when three trypanosomes were found. After thirty-five negative examinations they were again observed, about one-half per field, sixty-one days later, April 8. Three days later they increased to ten per field, after which they disappeared. Twelve examinations made between that date and May 27 were negative. On May 30 one organism was seen. An examination made four days later was negative, while one made June 6 showed parasites, about one-half per field. Fourteen examinations made between June 6 and July 29 were negative. On the latter date beginning lesions on the genitalia were noticed. August 1 (268 days after the first injection) the animal died in a condition of cachexia and infected with cocci, but no trypanosomes were seen. It will be seen that during this time trypanosomes were observed only five times.

Dec. 17	.....Last Inoc.	April 11	.....10 per field
Feb. 6	.....Two found	May 30	..... 1 found
April 8	.....1/2 per field	June 6	.....1/2 per field
		Aug. 1	.....died, 268 days.

Guinea-pig 5.105 was the only one to become infected, out of a set of ten which received ten injections each of ten cultures. These were given in the interval between November 26 and January 28. Seven days later, on February 4, a single trypanosome was observed, previous bi-weekly examinations being negative. The following thirty-four examinations likewise failed to reveal the parasite, which, however, was detected forty-six days later, on March 22 (about one-half per field). The blood was again negative two days later, but on March 26 and 28, and April 1 a few organisms were found. After two negative exam-

inations they were again found on the 11th, but were again missed on two subsequent occasions. Death occurred on April 19, or eighty-one days after the last injection, or 104 days after the first.

Jan.	28.....Last Inoc.	March 28.....	One per field
Feb.	4.....One found	April 1.....	Three found
March	22.....1/2 per field	April 11.....	One per field
March	26.....One found	April 19.....	died, 104 days.

The course of the infection in the last two guinea-pigs, when contrasted with that observed in the four animals which received only single injections, will be seen to be very mild and characterized by an infrequent occurrence of parasites. This peculiarity is undoubtedly due to the treatment with multiple injections as a result of which a mild degree of immunity was probably established. The remarkably long period in which no trypanosomes could be found suggests that the parasites localize and multiply in some other place than the blood stream, only occasionally appearing in the blood. That this is true in regard to rabbits and guinea-pigs will be shown later. It must be remembered, however, that parasites might be present in the blood in such small numbers as to make their detection by the ordinary examination of a fresh preparation quite impossible. Inoculation of the blood into the very susceptible rat would probably reveal the presence of an occasional trypanosome.

COURSE OF THE INFECTION IN RATS

The injection of seven-day cultures into rats as shown in Table 2 produces a fatal infection which, however, has a considerably longer duration than that produced by the virulent strain. The course may be subacute or chronic, the latter extending to 126 days. The injection of a twenty-one-day culture in one rat gave an unusually long duration of 265 days. As a rule, however, survival beyond fifty days is rather exceptional as will be seen from a study of the tables.

In the subacute type, the trypanosomes gradually increase in numbers until they are about fifty per field. The parasites are constantly present, though occasional remissions occur. In the more chronic cases the organisms at times apparently disappear from the circulation and the blood examinations may remain negative for a week or more. Thus in the rat which resisted 265 days, during a period of three months, the parasites were found only four times and then in very scanty numbers.

The long duration of the disease in rats is not due to the inhibitive action of antibodies, as might be inferred, but rather to the feebleness

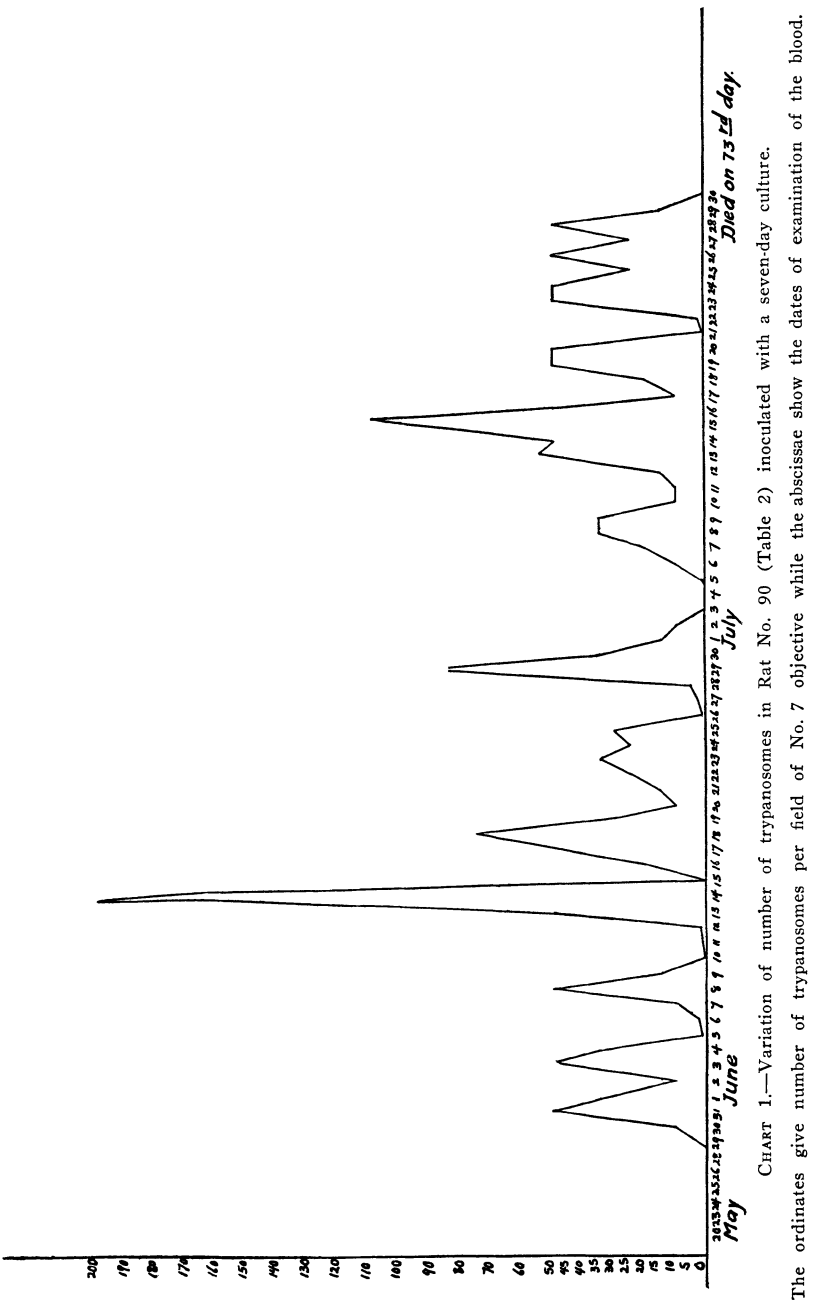

CHART 1.—Variation of number of trypanosomes in Rat No. 90 (Table 2) inoculated with a seven-day culture.

The ordinates give number of trypanosomes per field of No. 7 objective while the abscissae show the dates of examination of the blood.

or degree of attenuation of the organisms. The more attenuated an organism is, the longer it will take to restore its pathogenicity. At or shortly before death the trypanosomes have nearly regained their virulence, for on subinoculation, at such time, an acute infection is

TABLE 14

SHOWING RESULTS OF SUB-INOCULATIONS FROM EARLY AND LATE RAT INFECTION INTO RATS.

No. of Passage	Date of Inoculation	No. of Rat	Period of Incubation, Days	Death, Days
Early Infection. Started 11 days after Inoculation of Rat 3.22 with Culture				
1	Jan. 20	1.49*	—2	128
1	Jan. 20	2.49	—2	48
2	Jan. 23	3.49*	—3	36
2	Jan. 23	4.49	—3	24
3	Jan. 26	2.60*	—3	46
3	Jan. 26	3.60	—3	9
4	Jan. 29	5.49*	—2	25
4	Jan. 29	6.49	—2	9
5	Feb. 8	8.49*	—4	19
5	Feb. 8	9.49	—4	16
6	Feb. 14	5.98*	—2	39
6	Feb. 14	6.98	—2	25
7	Feb. 27	4.117*	—2	20
7	Feb. 27	5.117	—2	17
8	March 4	3.124	—2	21
8	March 4	4.124*	—2	27
9	March 13	5.141*	—2	12
9	March 13	6.141	—2	13
10	March 15	5.149*	—3	20
10	March 15	6.149	—3	11
11	March 21	5.164*	—4	11
11	March 21	6.164	—4	21
12	March 28	4.177*	—4	10
12	March 28	5.177	—4	9
13	April 6	3.190*	—2	14
13	April 6	4.190	—2	9
14	April 11	5.4	—4	11
14	April 11	6.4*	—4	10
15	April 20	5.19*	—2	5
15	April 20	6.19	—2	5
16	April 25	6.29		3
16	April 25	7.29		3
Late Infection. Started 35 Days after Inoculation of Rat 3.22; 5 Days before Death				
1	Feb. 13	4.92*	—3	9
1	Feb. 13	5.92	—3	9
2	Feb. 16	7.99*	—3	6
2	Feb. 16	8.99	—3	8
3	Feb. 22	1.108*	—4	5
3	Feb. 22	2.108	—4	5
4	Feb. 26	7.113	—2	4
4	Feb. 26	8.113	—2	5

* Sub-inoculations were made to the next following pair of rats from the animal marked

produced which by two or three consecutive passages completely restores the virulence so that death results in three to five days. Thus, Rat 1.49 (Table 14) was subinoculated on the day of its death into two rats which died in six and five days, respectively, and a second

passage into two rats reduced the duration to four days. Again, when a subinoculation was made from the same rat two days before death, the animal died in twelve days, and inoculation from the latter produced death in three and four days. On the other hand, subinoculations made from this original rat on the one hundred and second day, that is, twenty-six days before death, caused death in forty-five and ninety-seven days, respectively.

It will be seen from the foregoing that the trypanosomes which develop in the rat after injection of a culture remain attenuated for a considerable period, and that the final result is due to a rather sudden restoration of virulence. As further evidence of this fact reference is made to Table 14, which reproduces the results obtained by making subinoculations at the beginning and shortly before the end of the infection. Rat 3.22, which was used for this experiment, was injected with a seven-day culture on January 9. Trypanosomes appeared on the eleventh day (ten per field) and its blood was inoculated into the first set of two rats, 1.49 and 2.49. A series of successive passages, sixteen in number and extending over ninety-six days, were necessary to restore the virulence to a maximum. Had the subinoculations been made regularly, as soon as trypanosomes appeared in the blood, that is, every two or three days, it is probable that the attenuation of the strain would have been maintained for a much longer period. The second series of subinoculations was started five days before the death of the original rat, which occurred on the fortieth day (February 18) and three or four passages sufficed to restore the virulence.

Of special interest as indicating the attenuated character of the trypanosome present in the rat, after infection with a culture, is the result obtained by injecting guinea-pigs with blood drawn early and late in the course of the infection. Thus, Rat 5.72 received an injection of one seven-day culture, Generation 143, on January 17 and after a period of incubation of six to seven days trypanosomes appeared, three per field. Two drops of this blood, drawn from the tail, was injected into each of two guinea-pigs (Set A), Table 15. On February 13, the day before the death of the rat which occurred on the twenty-eighth day, two drops of its blood (thirty-five per field) were again injected into each of two guinea-pigs (Set B). As seen from Table 15, three guinea-pigs and probably four became infected after a very long period of incubation. Guinea-Pig 4.87, after a period of

incubation of forty-two to forty-six days, showed only a single trypanosome in the fresh blood preparation. Eleven subsequent bi-weekly examinations were negative, but two days before death the organism reappeared (two per field).

Examinations of Guinea-Pig 3.87 never revealed the parasite, but owing to their scarcity probably they were missed. Inasmuch as typical local lesions about the genitalia developed, this seems to have been the case and in all probability the animal became infected. At present (July 3, 1914) the guinea-pig is in excellent condition showing apparently healed lesions.

Guinea-Pig 5 and 6.161 (Set B, Table 15), after frequent examinations for a period of eight months, failed to show either a blood infection or skin lesions. Observations were then discontinued until about ten months after inoculation, when examination showed ulceration and alopecia around the genitalia. Trypanosomes were demon-

TABLE 15

SHOWING THE RESULTS OF SUB-INOCULATION FROM EARLY AND LATE RAT INFECTION INTO GUINEA-PIGS

Set	No. of Guinea-Pigs	Date of Inoculation	Period of Incubation Days	Results
A	3.87	Jan 24	.....	Apparently no infection; shows healed genital lesion. Alive 524 days (July 3, 1914).
	4.87	Jan 24	42-46	Died on ninetieth day.
B	5.161	Feb. 13	205-266	Posterior alopecia and ulceration of the genitalia. Died in about 300 days.
	6.161	Feb. 13	268-270	Posterior alopecia and ulceration of the genitalia. Died on 404th day.

strated in these lesions. One organism was found in the blood of Guinea-Pig 5.161 on one occasion (two hundrenth and sixty-sixth day). Four days later a similar observation was made on Guinea-Pig 6.161.

Guinea-Pig 5.161 died in December. Guinea-Pig 6.161 died on March 24, or 404 days after inoculation.

As shown, heretofore, only a very small number of guinea-pigs when injected with ten cultures, become infected. The greater insusceptibility of these animals is shown therefore in their behavior not only to cultures but also to the blood trypanosomes derived from such cultures.

Reference was made to the possibility of some localization of organisms, suggested by the long periods over which no organisms could be found in the blood stream. Cutaneous localizations of trypanosomes have been observed in the case of rabbits and guinea-pigs.

This condition seems to depend both on the natural resistance of the animal, and on the degree of virulence of the infecting organism. Thus, skin lesions have been produced in rabbits by inoculation of cultures, that is, very attenuated organisms and also of virulent trypanosomes. It is well known that rabbits have a relatively higher resistance to blood infections than guinea-pigs.

In case of guinea-pigs, no skin lesions, so far as we have observed, have resulted from inoculation of virulent organisms. On the other hand, an attenuated strain may be produced by inoculation of cultures into rats and when such organisms are inoculated into guinea-pigs, skin infection frequently results. As shown, these infections are characterized by very long duration and by the appearance of only occasional organisms in the blood.

Having shown that prolonged cultivation of the trypanosoma brucei results in an attenuation of the organism, which fact is seen either in a failure of such cultures to infect, or in an increased duration of the disease, it was of interest to determine the least infecting dose of virulent trypanosomal blood and compare this with that of the attenuated organism present in rat blood. Inasmuch as the results of this work will appear in another paper it will be sufficient at present to state that while the minimum infecting dose of the normal virulent strain for rats is represented by a single organism, at least three of the attenuated trypanosomes are necessary for infection. In guinea-pigs, one normal trypanosome is sufficient to infect, while the attenuated strain, as just shown, yields very inconstant results, when many hundreds or even thousands of organisms are injected.

SUMMARY

Trypanosoma lewisi gradually loses its virulence on cultivation, and is practically non-infective in the seventy-fifth generation.

A dialyzed nutrient agar, plus serum (1 to 2) yields the most constant results in isolation of trypanosoma brucei. The trypanosoma brucei is capable of growing on hemoglobin-free medium.

The continued cultivation of trypanosoma brucei markedly attenuates the parasite, the latter becoming avirulent, except in few cases,

for guinea-pigs and rabbits, and less virulent for rats, mice and dogs. In time, probably a wholly avirulent strain can be obtained.

A single culture, seven days old, as yet invariably infects the rats, but when kept at room temperature for an additional one, two or three weeks it becomes less infective or not at all. Multiple injections of such aged cultures in some cases produce infection. The non-virulent cultures, twenty-eight days old, are still capable of growth in vitro.

Cultures induce a subacute or chronic infection, the longest survival in rats being 265 days; in guinea-pigs, 268 days.

The blood of rats infected with cultures is infective for guinea-pigs after a very long period of incubation, and the guinea-pigs develop marked local lesions, especially about the genitalia. Trypanosomes can be demonstrated in these lesions.

Consecutive passage of the attenuated strain through rats restores the virulence. This is accomplished more readily when the first passage is made with blood drawn shortly before death.

The successful production of an attenuated strain opens the possibility of immunizing animals against infection with trypanosoma brucei.

I wish to express my sincere gratitude to Professor F. G. Novy for his constant interest, advice, and many valuable suggestions during the pursuance of this work.